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14. ABSTRACT
Abundant epidemiological and experimental evidence establishes alterations in cholesterol metabolism as a key driver of prostate cancer (PCa) aggressiveness. Our preliminary data shows cholesterol sulfotransferase (SULT) 2B1b, a global regulator of cholesterol metabolism, is overexpressed in human prostate neoplasia and PCa cell lines and that genetic knock down suppresses LNCaP growth and diminishes androgen receptor (AR) activity. It is hypothesized that SULT2B1b modulates PCa growth and phenotype via alterations in cholesterol metabolism. If validated, the studies will form the foundation for novel therapeutic intervention.

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Title: Targeting Sulfotransferase (SULT) 2B1b as a Regulator of Cholesterol Metabolism in Prostate Cancer

Grant number: W81XWH-14-1-058830

Rationale: Abundant epidemiological and experimental evidence establishes alterations in cholesterol metabolism as a key driver of prostate cancer (PCa) aggressiveness. Therapeutically targeting cholesterol metabolism in PCa through the use of cholesterol-lowering drugs (statins) decreases the occurrence of aggressive PCa. Our preliminary data show cholesterol sulfotransferase (SULT) 2B1b, a global regulator of cholesterol metabolism, is highly expressed in many clinical PCa specimens and PCa cell lines and that genetic knock down suppresses LNCaP growth and diminishes androgen receptor (AR) activity. It is hypothesized that SULT2B1b modulates PCa growth and phenotype via alterations in cholesterol metabolism. If validated, the studies will form the foundation for novel therapeutic intervention.

Hypothesis or objective: The primary objective of these studies is to define the role of SULT2B1b in PCa on growth, invasion, and sensitivity to androgens, and apoptosis in androgen responsive and non-responsive phenotypes based on the central **hypothesis that SULT2B1b regulates malignant phenotypes via regulation of cholesterol metabolism**. The pivotal role of SULT2B1b in regulating cholesterol homeostasis in PCa under standard growth conditions is a novel observation that when better defined by the studies outlined in this application, will provide the foundation for new approaches for controlling cholesterol dysregulation in PCa. To better define the role of SULT2B1b in PCa, the following goals are set forth herein: a) to validate the role of SULT2B1b in modulating cholesterol dysregulation, b) to elucidate the pathway(s) by which SULT2B1b modulates cholesterol levels in PCa, and c) to elucidate the mechanism(s) of SULT2B1b-mediated AR activity regulation. In addition, the potential role of SULT2B1b on regulating biosynthetic pathways associated with de novo androgen synthesis will be addressed based on the **hypothesis that SULT2B1b promotes PCa proliferation by impacting the biosynthesis and metabolism of androgen.** To accomplish these goals, the following specific aims are proposed:

Research Approach:

Aim 1. To elucidate mechanism(s) by which SULT2B1b modulates cholesterol metabolism. Stable and tetracycline (tet)-inducible PCa cell lines (LNCaP, VCaP, PC3, and DU145) expressing shRNA (short hairpin RNA) against SULT2B1b or full-length SULT2B1b cDNA have been developed and verified and will be used to assess impact of SULT2B1b modulation on PCa. Studies in this aim address the ***hypothesis that SULT2B1b-mediated sulfonation of oxysterols and/or*** SREPB-2 ***is central to cholesterol dysregulation in PCa via limiting their agonistic effects on LXR*** and will a) verify that modulation of SULT2B1b alters cholesterol levels in PCa cells; b) identify SULT2B1b-mediated signals that modulate LXR activity; c) evaluate SULT2B1b modulation of cholesterol homeostasis via the non-LXR-mediated pathway in which SULT2B1b controls cholesterol biosynthesis by direct regulation of SREBP-2 activity.

Aim 2: To elucidate mechanism(s) of SULT2B1b-mediated alterations in apoptotic and AR responses. Studies in this aim address the ***hypothesis that SULT2B1b modulates PCa growth via alterations in AR response that may or may not impact response to apoptotic stimuli***. These studies will indentify SULT2B1b-mediated signals that alter AR activity via crosstalk between LXR and AR and regulation and function of AR co-factors.[11] Also, the impact on androgen concentration, AR expression, the binding of androgen to AR, the subcellular localization of AR, and the binding of AR to target gene promoters, and activation of the AR transcriptional activity will be assessed. These studies will also assess SULT2B1b impact on apoptosis by activation of death receptors via exposure to recombinant TRAIL, Fas Ligand and TNFα proteins.[18]

Aim 3: Validating the biological function of Sult2B1b via chemical probes. RNAi knockout of genes to probe biological function of a target protein is an important first step in probing biological function but it has its limitations due to disruption of all protein functions confounding interpretation of the importance of the catalytic activity. An alternative approach to studying the importance of the catalytic activity of an enzyme to its biological function is to utilize small molecule inhibitors. Therefore, we will develop small-molecule inhibitors of SULT2B1b to probe its biological function. We developed a new fluorescence assay of Sult2B1b activity that can be used to determine the inhibitory potency of compounds and to perform high-throughput screening of compound libraries. We have also identified potential inhibitors via *in silico* screening using the available X-ray structure of SULT2B1b. We propose to use this new assay to determine the inhibitor potential of these compounds and to then test the best inhibitors for their ability to inhibit the formation of cholesterol sulfate in cell culture. We will then test the anti-proliferative activity of these inhibitors and will compare the results to those obtained via siRNA.

Accomplishments.

Major Task 1: generation of SULT2B1b modulated PCa cell lines: Model development :
Subtask 1: Develop lentiviral plasmids containing inducible shRNA targeting SULT2B1b and SULT2B1b cDNA, under control of inducible Tet-a promoter vectors used include pSlik and/or PLNKO (obtained from Addgene) , pLenti6 (purchased from Invitrogen),and pLenti-X (from Celontech) , Subtask completed? Completed and reported in the previous progress report (2014-1015).
Subtask 2: Production of infective Lentivirus using co-transfection of HEK293 (pCa cells LNCaP, PC3, DU145, and VCaP (purchased from ATCC) with lentivirus plasmids generated in Subtask1 and accessory lenitivirus plasmids (purchased from Clontech and Addgene) Subtask completed?: Yes, data contained in prior progress report (2014-2015) day) lentivirus transduction.

Subtask 3: **Test function of lentivirus-mediated, tet-inducible SULT2B1 overexpresssion or endogenous knock down by transient transduction using RT-PCR and western blotting in PCa cell lines LNCaP, PC3, and DU145 (all from ATCC).** Subtask completed, see description above.
Stable transduction, antibiotic selection and validation of tet-inducible modulation of sult2b1b. Complete and contained in previous pr
Major Task2: Characterize impact of SULT2B1b modulation on lipid metabolism in human PCa cell lines

Subtask 2: Contact Roswell Park shared resource to arrange for sample analysis. Determine requirements for analysis: siRNA-based SULT2b1knock down in LNCaP and shipment to Roswell Park was reported in the last progress report. The data have been returned but no androgen was detected in any sample. The issue was discussed with Roswell Park investigators after which the approach was abandoned.

Cell lines used: wt LNCaP, VCaP (from ATCC), and PCa lines with Tet-regulated SULT2b1b created in major task 1], because we were unable to produce the stable clones, we substituted transient siRNA knock-down PCa cells for the in vitro cellular analysis

Subtask 3: Characterize SULT2B1b as a regulator of PCa growth by modulating SULT2b1b in PCa cell lines and measuring tumor growth

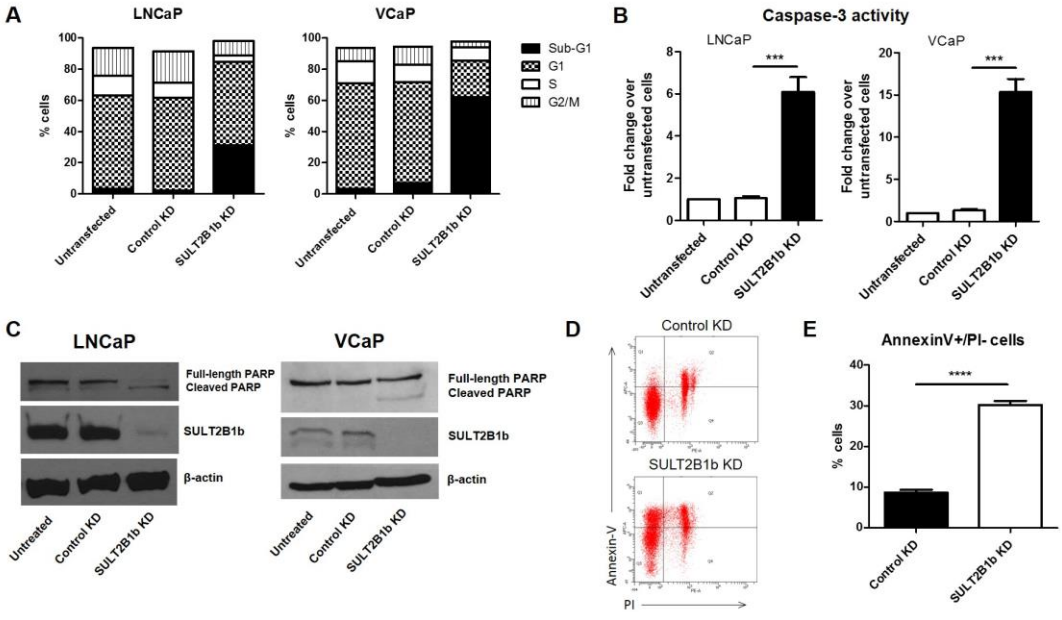
Cell lines used: : Cell lines used: wt LNCaP, VCaP (from ATCC), PC-3, DU145, C4-2 and RWPE-1. SULT2B1b in the cell lines was modulated with transient SULT2B1 modulation using siRNA as described in the previous progress report. The PCa lines with Tet-regulated SULT2b1b created in major task 1 (PC-3 and DU145) were assessed for impact of overexpression of SULT2B1b as part of the validation linking SULT2B1b to cholesterol sulfate.

Major Task 2: In Vitro testing/growth assays with PCa cell lines (Specific aim 1: To establish the critical role of SULT2B1b activity in PCa growth and phenotype *in vitro*. (Months 4-24)

- Subtask 1: Cell proliferation assays. Reported in last progress report (2015)
- Clonogenic/plating efficiency assays. (reported in last progress report (2015)
- Soft agar growth assays. Reported in last progress report (2015)
- LXR activity assays. – Reported in last progress report (2015)
- Akt phosphorylation assays – This assay was not performed because the data led the project in a different direction.

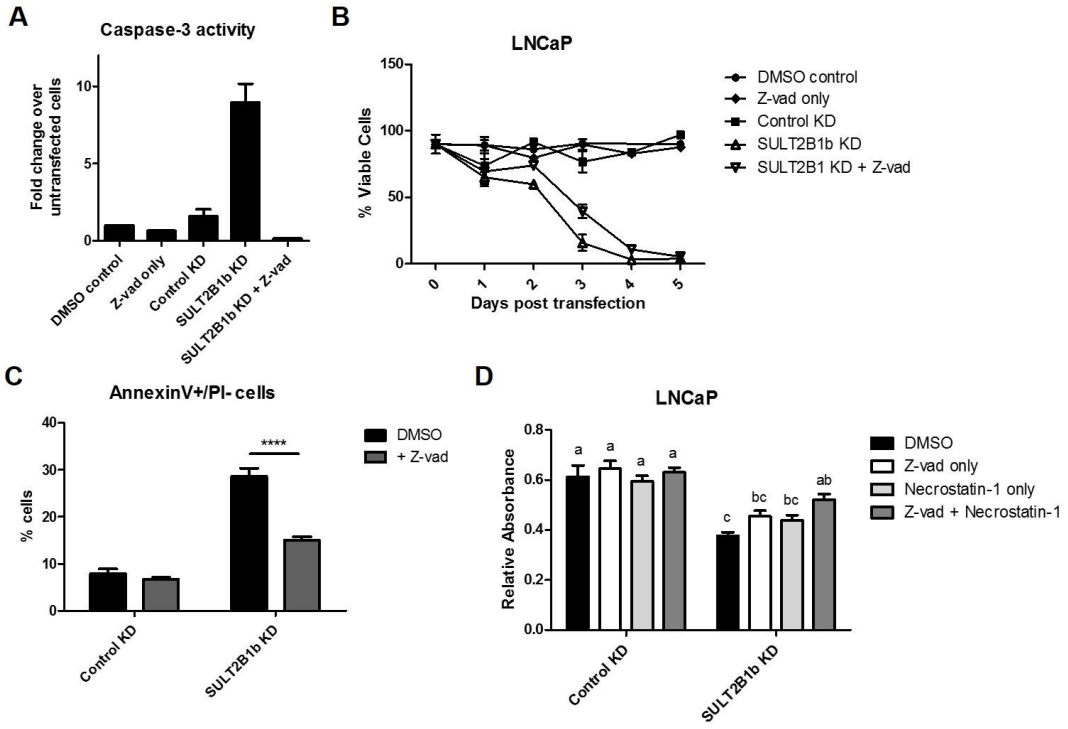
Based in the data provided in the 2015 progress report demonstrating the induction of cell death upon siRNA knock down of SULT2B1b, studies were performed to assess to assess the mechanism of cell death (Figure 1).

Figure 1. SULT2B1b KD induces cell death in PCa cells. LNCaP and VCaP cells were transfected with control or SULT2B1b siRNA and harvested after 72 hours. **A)** Cell cycle analysis of LNCaP (left) and VCaP (right) cells. Cell samples were analyzed by flow cytometry after fixing and staining with propidium iodide (PI). Data is representative of three independent experiments. **B)** Caspase-3 activity in LNCaP (left) and VCaP (right) cells was determined by luminescence assay after control or SULT2B1b siRNA KD. Results indicate the mean ± SEM of three independent experiments. **C)** Western blots of indicated proteins after control or SULT2B1b siRNA transfection in LNCaP (left) and VCaP (right) cells. **D)** A representative sample of LNCaP cells after incubation with control or SULT2B1b siRNA Annexin-V and PI. Cells were harvested and stained before analysis by flow cytometry. **E)** Summary of replicate samples shown in **(D)**. Data represents the %Annexin-V+/PI- cells out of the total population of events (excluding doublets) and is representative of three independent experiments performed in duplicate.



After observing that SULT2B1b KD decreased cell viability in multiple cell lines, further investigation showed that SULT2B1b induced cell death in LNCaP and VCaP cells (previous progress report). SULT2B1b KD increased the percentage of sub-G1 nuclei by cell cycle analysis and significantly increased caspase-3 activity and PARP cleavage in both cell lines (Figure 1A-C). Increased sub-G1 nuclei percentages and caspase-3 activation was also observed in C4-2 cells with SULT2B1b KD (not shown). Furthermore, LNCaP cells with SULT2B1b KD analyzed by flow cytometry showed an increased percentage of Annexin-V+/propidium iodide (PI)- cells compared to control KD cells (Figure 1D-E). These data suggest that SULT2B1b KD induces apoptosis via caspase activation. To determine whether caspase activation

Figure 2. Cell death resulting from SULT2B1b KD persists with pan-caspase inhibition. A) Caspase-3 activity in LNCaP cells at 72 hours after transfection. Bars indicate the mean±SEM of duplicate experiments. **B)** Cell viability determined via trypan blue cell counting in LNCaP cells at indicated time points after control or SULT2B1b siRNA KD with and without addition of 20μM pan-caspase inhibitor, Z-vad. **C)** LNCaP cells were treated with 20μM Z-vad at the time of control or SULT2B1b siRNA transfection. After 72 hours, cells were harvested, incubated with Annexin-V and PI, and analyzed by flow cytometry. Bars represent the mean ± SEM of %Annexin-V+/PI- cells within total events collected (excluding doublets) from three independent experiments. **D)** Graph of MTS assay endpoint analysis in LNCaP cells after 96 hours of Control or SULT2B1b siRNA KD as well as treatment with 20μM Z-vad and/or 10μM Necrostatin-1. Z-vad was added every 24 hours and Necrostatin-1 was added every 48 hours in applicable samples. Bars represent the mean +/- SEM of three independent experiments. Statistical analysis was conducted using two-way ANOVA and Tukey's post-test. Different letters indicate significant differences among treatments.

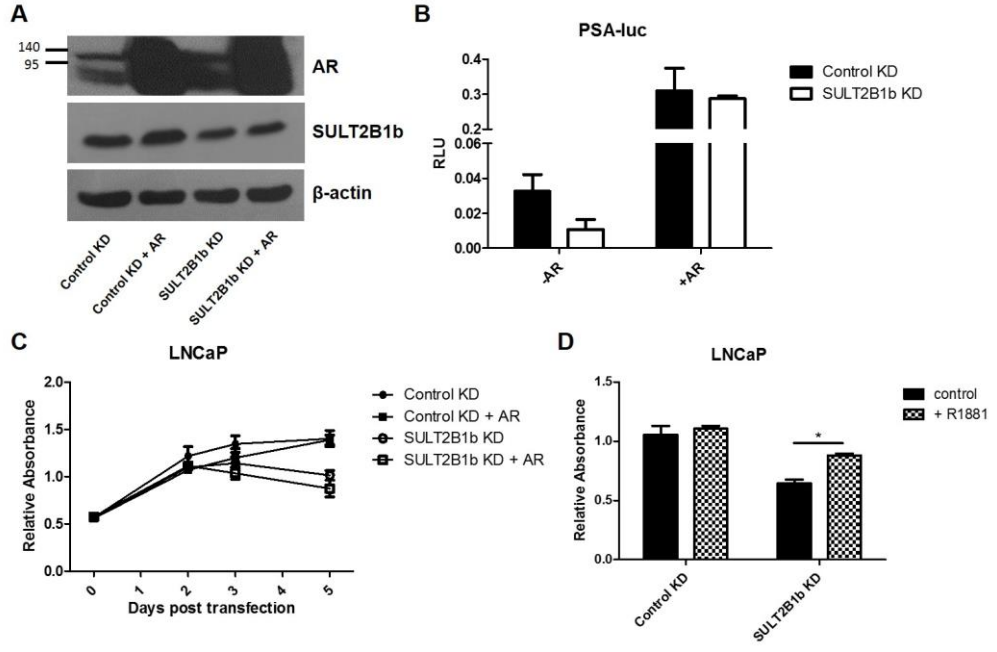


was an essential aspect of the induced cell death, abrogation of apoptosis by the addition of the pan-caspase inhibitor, Z-vad-fmk (Z-vad), was tested in SULT2B1b KD LNCaP cells. The addition of Z-vad successfully blocked caspase-3 activation and abrogated the enhancement of Annexin-V+/PI- cells after SULT2B1b KD, however,

LNCaP cell viability and, ultimately, cell death was not altered (Figure 2A-C). Thus, LNCaP cells with SULT2B1b KD in the presence of Z-vad were able to induce cell death using alternate mechanisms. While the complete mechanism(s) of cell death are not understood, it is possible that after pan-caspase inhibition SULT2B1b KD cells activated alternative cell death pathways. Additional studies completed in SULT2B1b KD cells with co-treatment of Z-vad and an additional inhibitor of RIP1 kinase, Necrostatin-1, yielded a greater proportion of viable cells compared to Z-vad treatment alone (Figure 2D). It may be of note that although LNCaP cells with SULT2B1b KD respond to Necrostatin-1 treatment with concurrent caspase inhibition, our studies investigating reactive oxygen species production and AnnexinV-/PI+ cells did not show that RIP1 kinase-dependent death pathways (i.e. during necroptosis(21)) are directly induced by SULT2B1b KD (data not shown).

In the last progress report, we showed that SULT2B1b knock down modulated androgen receptor (AR). Further studies were performed to confirm the initial observation (Figure 3).

Figure 3. AR ligand addition, but not AR overexpression, partially rescues cell growth in LNCaP with SULT2B1b KD. A) Western blot showing expression levels of indicated proteins. Cells were harvested 60 hours after transfection with Control or SULT2B1b siRNA. **B)** Luciferase assay showing AR activity after 72 hours of siRNA transfection. **C)** MTS assay of LNCaP cells with or without AR overexpression followed by Control or SULT2B1b siRNA transfection. The time of siRNA transfection was considered Day 0 and relative absorbance was identified at the indicated time points. Each point represents the mean +/- SEM of 3 replicate wells. **D)** 72 hour end point analysis from an MTS assay of LNCaP cells that were transfected with control or SULT2B1b siRNA with or without addition of 10nM R1881 daily. Each bar represents the mean +/- SEM of 3 replicate wells and similar results were found in duplicate experiments. * indicates $p<0.05$ determined by two-way ANOVA.



Further investigation showed that the decreased expression of the AR mediated by SULT2B1b KD was not the cause of decreased cell growth, since transient overexpression of the AR in LNCaP cells with SULT2B1b KD showed no increase in cell growth over LNCaP cells with SULT2B1b KD alone (Figure 3). Overexpression of the AR resulted in increased AR activity in both Control KD and SULT2B1b KD samples (Figure 3B), however, this elevation in AR activity did not rescue the decreased cell growth in SULT2B1b KD cells (Figure 3C). Finally, a growth assay of LNCaP cells shows that replenishing the culture medium with synthetic androgen, R1881, partially, yet significantly, rescues the reduced cell growth in SULT2B1b KD LNCaP cells back to Control KD levels (Figure 3D).

Taken together these data demonstrate that SULT2B1b is an important enzyme in prostate cancer. Its removal generates apoptosis in androgen responsive as well as castration resistant cell lines. The data reported in the previous progress report in combination with the data reported herein resulted in manuscript that was published in 2016 (Vickman, R. et al. Mol Cancer Res 14:776, 2016. PMID:27341831)

Major Task 3: In vivo xenograft model (Months 4-36,)

Subtask 1: Establish xenograft model, preliminary tumorigenic studies to establish basic parameters needed for statistical validation of the tumor studies including implantation efficiency

Subtask 2: Implantation of stable cell lines produced in task 1, and monitoring of survival in mice. As reported in the previous progress report, these studies will not be pursued because we are able to substitute transient siRNA knock-down in all PCa cell lines for the in vivo studies. We are working to generate small molecule inhibitors to perform the in vivo tumor inhibition studies.

Major Task 4: **Development of small molecule SULT2B1b inhibitor: Testing of identified lead compounds**

- ***Subtask 1:*** Assessment of compound effects on SULT2B1b enzyme activity of cholesterol sulfate production in vitro.

We described the development of a coupled-enzyme assay to measure the activity of Sult2B1b in the previous progress report (2015).

- ***Subtask 2:*** Confirm specificity by evaluation of sulfonation activity on other sterols using in vitro assays.

Seven thousand (7,000) compounds were screened after computational docking studies identified potential inhibitory compounds. The results were described in the previous progress report where we observed 3 confirmed hit compounds. These hits were not ideal for further development. Thus an additional 20,000 compounds were screened after further optimization of the assay. In evaluating the initially identified hits, we were able to further optimize the process. First, the non-specific targeting for the coupling enzyme

SULT1A1 was identified. To achieve a better differentiation of screened compounds' effects on SULT2B1b and the coupling enzyme, an additional screening for SULT1A1 has been included. In the activity assay for SULT1A1, its substrates, 20 μM PAP and 400 μM MUS, are provided for 2 μM SULT1A1. The other conditions remain the same as in the coupled-enzyme assay. Similarly, the increase of fluorescence intensity due to the accumulation of MU is used to reflect SULT1A1 activity. Since high-purity PAP is provided instead of a PAPS fragment in the first step of coupled-enzyme assay, this SULT1A1 activity assay significantly increases the dynamic range, thus improves our ability to identify the effects of compounds on SULT1A1. Meanwhile, the coupled-enzyme assay is performed and compared with the results from SULT1A1 screening. Using the improved screening assay, 32 compounds were identified as the initial hits by the high throughput screen, providing a hit rate of 0.16%. Similar to the confirmation process in the previous round, we have taken small amount of compound solution from the 384-well screening plates and 96-well mother library plates to confirm the hits. The ones showing the most consistent results have been ordered in powder form. When they are received, further validation will be pursued.

Impact.

Innovation: Preliminary studies highlight an important and novel role for SULT2B1b as a pivotal regulator of cellular cholesterol metabolism in PCa. The studies outlined in this application employ standard analysis methods; however, the conceptual hypothesis that SULT2B1b expression in PCa is central to dysregulation of cholesterol homeostasis is innovative. While a major focus of PCa research is on the AR and its impact on PCa growth, the proposed studies will probe the impact of SULT2B1b-mediated sulfation on pathways regulating cholesterol metabolism, including LXR and alternate non-LXR pathways. If validated, the studies will provide the foundation for developing novel approaches to controlling cholesterol dysregulation in PCa, which is known to contribute to AR activity as well as apoptosis and cellular proliferation. Thus, **establishing SULT2B1b as a key regulator of PCa growth and progression could potentially establish SULT2B1b a novel therapeutic target.**

Impact/ Overarching Challenges and focus area. Cholesterol sulfate (CS) expression in prostate neoplasia appears to correlate to SULT2B1b activity suggesting a potential role of CS as a **biomarker** of prostate cells with altered growth rate and response to androgens. By better understanding how SULT2B1b 1) controls alterations in cellular responses and 2) regulates activity of immunosuppressive, tumor-derived factors such as oxysterols, implicate a role for SULT2B1b in prostate **tumor and microenvironment biology**. Validation of SULT2B1b as key controller of cholesterol dysregulation may also lead to development of better **therapeutics** for the treatment of both organ-confined disease and **advanced prostate cancer**.

The studies proposed in these specific aims will be performed as accomplishing the following tasks as outlined in the approved statement of work (SOW). The following is a description of activities and accomplishments in the first year of the project:

Changes/Problems.

The discovery of the induction of apoptosis after SULT2B1b knockdown has led to the use of single cell RNA-seq on normal LNCaP cells compared to SULT2B1b knockdown LNCaP. These studies have been performed and we are analyzing the data with the intent of obtaining an unbiased pathway analysis that will aid us in understanding the impact of loss of SULT2B1b.

Products.

Published Manuscript

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Molecular Cancer Research

Cholesterol Sulfonation Enzyme, SULT2B1b, Modulates AR and Cell Growth Properties in Prostate Cancer

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Abstract

Cholesterol accumulates in prostate lesions and has been linked to prostate cancer incidence and progression. However, how accumulated cholesterol contributes to prostate cancer development and progression is not completely understood. Cholesterol sulfate (CS), the primary sulfonation product of cholesterol sulfotransferase (SULT2B1b), accumulates in human prostate adenocarcinoma and precancerous prostatic intraepithelial neoplasia (PIN) lesions compared with normal regions of the same tissue sample. Given the enhanced accumulation of CS in these lesions, it was hypothesized that SULT2B1b-mediated production of CS provides a growth advantage to these cells. To address this, prostate cancer cells with RNAi-mediated knockdown (KD) of SULT2B1b were used to assess the impact on cell growth and survival. SULT2B1b is expressed and functional in a variety of prostate cells, and the data demonstrate that SULT2B1b KD, in LNCaP and other androgen-responsive (VCaP and C4-2) cells, results in decreased cell growth/viability and induces cell death. SULT2B1b KD also decreases androgen receptor (AR) activity and expression at mRNA and protein levels. While AR overexpression has no impact on SULT2B1b KD-mediated cell death, the addition of exogenous androgen is able to partially rescue the growth inhibition induced by SULT2B1b KD in LNCaP cells. These results suggest that SULT2B1b positively regulates the AR either through alterations in ligand availability or by interaction with critical coregulators that influence AR activity.

Implications: These findings provide evidence that SULT2B1b is a novel regulator of AR activity and cell growth in prostate cancer and should be further investigated for therapeutic potential. *Mol Cancer Res; 14(9); 776–86. ©2016 AACR.*

Introduction

Prostate cancer is the second leading cause of noncutaneous cancer death among males in the United States, with 180,890 new cases and 26,120 deaths estimated in 2016 (1). Current treatments for organ-confined disease, including prostatectomy and radiation, have proven to be successful. Unfortunately, the initial treatment for metastatic disease, androgen-deprivation therapy (ADT), is palliative, providing only approximately 11 months of failure-free survival (2). Progression leads to the development of castration-resistant prostate cancer (CRPC) for which second-generation ADT is available, but the response is also short-lived (3, 4). Further investigation into the biology of prostate cancer and identification of novel regulators of cellular growth and/or androgen receptor (AR) activity is required for alternate, more curative therapeutic options for men with advanced prostate cancer.

Cholesterol metabolism dysregulation has been investigated in prostate cancer for many years, leading to a better understanding of cholesterol's contribution to disease progression. Through modulation of intracellular signaling and prosurvival pathways, cholesterol metabolism is believed to play a major role in prostate cancer development and progression to the castration nonresponsive state, but the mechanisms involved remain elusive (5–9). A number of modified forms of cholesterol are found within cells, namely, hydroxysterols, cholesterol esters, and cholesterol sulfate (CS). Previously, desorption electrospray ionization mass spectrometry (DESI-MS) was used to identify unique lipid profiles in human prostate tissue specimens, where CS was observed to be elevated in prostatic intraepithelial neoplasia (PIN) and adenocarcinoma lesions compared with normal tissues (10). This suggests that the enzymes responsible for converting cholesterol to CS are not simply expressed, but functional in these tissues.

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Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

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Cytosolic sulfotransferases (SULT) are a class of enzymes that catalyze the sulfonation of various hydroxysteroid substrates. SULT members within this class each have preferential affinity for various substrates. For example, SULT2A1 is expressed in the liver and adrenal gland, where it utilizes dehydroepiandrosterone (DHEA) as a substrate to produce and secrete the abundant circulating hormone precursor, DHEA-sulfate; SULT2A1 has been studied in prostate cancer as a result of this function (11). SULT2B1 has two isoforms, SULT2B1a and SULT2B1b, which differ only by 15 amino acids at the N-terminus due to alternate transcriptional start sites of the same gene. SULT2B1a and SULT2B1b each have different substrate affinities (12). SULT2B1b, the "cholesterol sulfotransferase," is expressed in a variety of tissues, including the prostate, placenta, and skin, but it is unclear whether SULT2B1a is expressed at the protein level in any human tissues (12–14). SULT2B1b-catalyzed production of cholesterol sulfate (CS) is critical for skin barrier layer formation and proper membrane function of a variety of cell types (13, 15). SULT2B1b is also known to influence cell metabolism through regulation of the Liver X Receptor (LXR), a global regulator of cholesterol homeostasis (16).

Studies have shown a potential role of SULT2B1b in the growth and progression of cancer cells (17–20). SULT2B1b was studied in prostate cancer in androgen-free conditions supplemented with DHEA (19, 21). However, SULT2B1b has not been previously evaluated in androgen-replete conditions that mimic normal biology and prostate cancer progression, consistent with the conditions in which CS accumulation was observed (10). Here, we investigate the impact of SULT2B1b in prostate cancer cells in the presence of androgen with the hypothesis that enzymatic activity of SULT2B1b supports cell growth and contributes to progression of the disease through modulation of LXR and AR activity. Studies reported herein verify that SULT2B1b is an important metabolic enzyme in multiple prostate cancer cell lines by demonstrating that SULT2B1b knockdown (KD) induces caspase-3 activation and cell death. Additionally, data show that SULT2B1b activity positively correlates with AR activity in prostate cancer cells. This positive regulation of AR by SULT2B1b appears to be LXR-independent and may be due to alterations in AR expression levels or ligand availability, because replenishing testosterone partially overcomes the growth-inhibitory effects of SULT2B1b KD. These studies support a novel mechanism of AR regulation in prostate cancer cells. Thus, therapeutic targeting of SULT2B1b may be a novel approach to inhibiting AR activity in prostate cancer.

Materials and Methods

Human prostate tissue source and immunohistochemistry

A random selection of four unpaired and four paired frozen, human prostate tissue samples were obtained from the Indiana University Simon Cancer Center Tissue Bank. All tissues were handled in accordance with the Indiana University institutional review board and prepared as previously described (10). Frozen sections were prepared on slides and subjected to hematoxylin and eosin (H&E) staining and pathological evaluation.

For SULT2B1b immunohistochemistry (IHC), slides containing frozen tissue specimens were rinsed with ddH_20 and subjected to the following treatments: 3% hydrogen peroxide, protein block solution (#X0909, Dako), 1:500 anti-SULT2B1 primary antibody (ab88085, Abcam), peroxidase-linked polymeric anti-mouse antibody (K4006, Dako), and 3,3′-diaminobenzidine (Dako). Samples were washed between each step, and Gills II hematoxylin was used as a counterstain.

DESI-MS

A laboratory-built DESI ion source, similar to the commercial 2D source from Prosolia, Inc. was coupled to a linear ion trap mass spectrometer (LTQ) controlled by XCalibur 2.0 software (ThermoFisher Scientific) and used in all experiments. The negative ionization mode was used with the automatic gain control (AGC) inactivated. Tissues were analyzed as previously described (10). For cell lines, the spray solvent used for DESI-MS was dimethylformamide (DMF)-acetonitrile (ACN) at a 1:1 ratio in volume; both solvents were purchased from Mallinckrodt Baker Inc. and infused over the cell line material at 1.0 μL/min flow rate through the instrument syringe pump. The DESI source parameters are indicated in Supplemental Materials and Methods. Each cell line was analyzed by manual movement of a 2D moving stage where the slide was held in a fixed position. Full-scan mass spectra were acquired in negative ion mode in the mass range m/z 200–1,000.

Cell lines

LNCaP, VCaP, RWPE-1, PC-3, and DU 145 were purchased from the American Tissue Culture Collection (ATCC) and maintained in media conditions identical to those recommended by ATCC. C4-2 cells were a generous gift from MD Anderson (Houston, TX) and were maintained in T-medium (Invitrogen) supplemented with 10% FBS and 1% streptomycin/penicillin. Cell lines were verified using cell line authentication testing from DDC Medical through the generous support of the Prostate Cancer Foundation and results are shown in Supplementary Table S1. All experiments were completed within 20 passages of acquisition from ATCC.

RNA interference (RNAi)

For siRNA KD, *SULT2B1* (HSC.RNAI.N004605.12.2), *NR1H2* (LXRβ, HSC.RNAI.N007121.12.2), and *NR1H3* (LXRα, HSC.RNAI.N005693.12.2) predesigned DsiRNA duplexes were purchased from IDT. Nontargeting siRNA was purchased as a control (Dharmacon Scientific). Transfection using Lipofectamine RNAiMax (Invitrogen) was completed for all siRNA transfections according to the manufacturer's instructions. For double siRNA KD, combinations of the same amount of siRNA were incubated with double the recommended transfection reagent prior to adding to cells in culture. "Controlx2" indicates twice the amount of nontargeting siRNA and double RNAiMax compared with "Control" samples. For shRNA KD in LNCaP cells, SULT2B1 and control sequences 5′-CCTCTATCATTACTCCAAGAT-3′ (LNKD) and 5′-CCATTAACTCTTTCCCGAAAT-3′ (LNCon), respectively, were inserted into the pLNKO.1 Tet-ON vector (Addgene) and transfected using FuGene HD (Promega). Finally, SULT2B1 shRNA and copGFP Control Lentiviral particles (Santa Cruz Biotechnology, Inc. sc-44399-V and sc-108084, respectively) were transduced in LNCaP cells using Polybrene (Santa Cruz sc-134220). The appropriate method of SULT2B1b KD is indicated in figure legends.

RNA isolation, cDNA amplification, and qRT-PCR

Total RNA was isolated using the E.Z.N.A. Total RNA Kit I (Omega Biotek) according to the manufacturer's instructions.

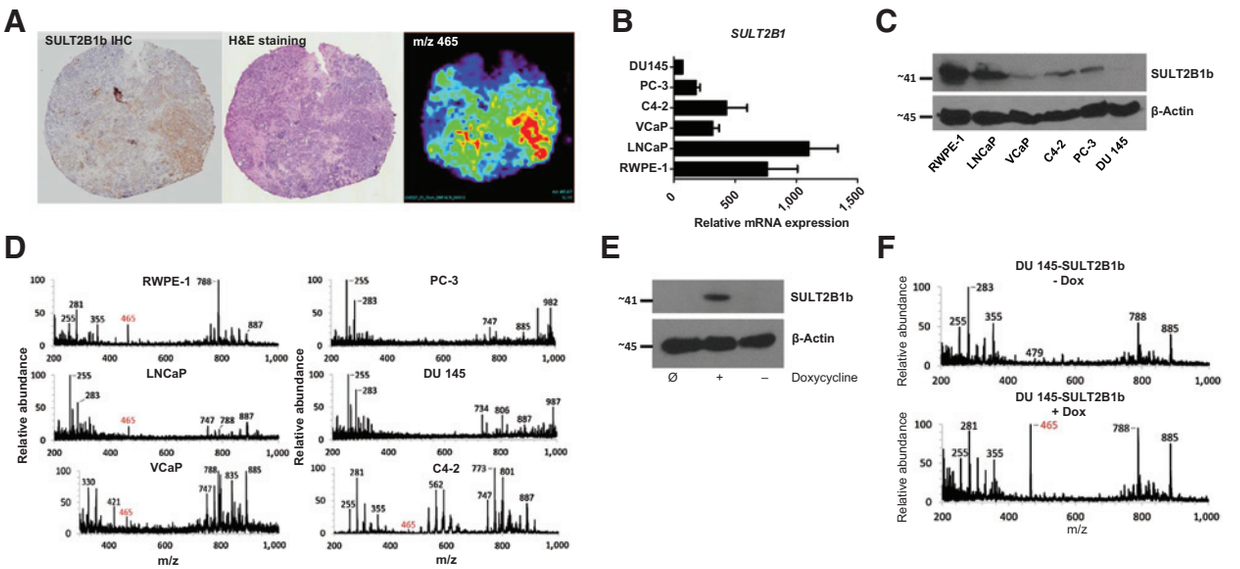

Figure 1.

CS accumulation correlates with SULT2B1b expression in prostate cancer cells and human prostate specimens. **A,** analysis of human prostate cancer by IHC for SULT2B1b (left), H&E staining (middle), and DESI-MS detection of CS at m/z 465 (right). These sections were derived from one representative patient out of a sampling of 8 patients. **B** and **C,** basal SULT2B1b expression was determined in indicated prostate cell lines by qRT-PCR (normalized to 18s rRNA) (**B**) and Western blot (**C**). Error bars represent the mean ± SEM of 3 replicate analyses. **D,** DESI-MS spectra of prostate cell lines from (**B–C**). CS expression is indicated by a peak at m/z 465 (indicated in red). **E** and **F,** stable DU145-SULT2B1b cells were treated with or without 1 μg/mL doxycycline for induction of SULT2B1b cDNA expression and analyzed by DESI-MS. **E,** a Western blot detecting SULT2B1b and β-actin in wild-type DU145 (Ø), DU145-SULT2B1b +Dox, and DU145-SULT2B1b –Dox is shown. **F,** DESI-MS-negative ion spectra in DU145-SULT2B1b ± Dox cells from (**E**) indicate the relative abundance of CS in cells by a peak at m/z 465.

cDNA was prepared by mixing 2 to 4 μg total RNA, 250 μmol/L dNTPs (Amresco), 0.5 μmol/L each of random hexamer and oligo(dT)$_{15}$ primers (Promega), and 200 units M-MLV reverse transcriptase with included reaction buffer(NEB). qRT-PCR was conducted using PerfeCTa FastMix II (Quanta Biosciences) according to the manufacturer's instructions. PrimeTime qRT-PCR gene probes (IDT) used for these studies include *ABCG1* (Hs.PT.56a.20848083.g), *AR* (Hs.PT.56a.38770693), *KLK3*, which will be identified herein as "PSA" (Hs.PT.56a.38546086.g), *NR1H2* (Hs.PT.56a.45297581.g), *NR1H3* (Hs.PT.56a.40638751.g), and *SULT2B1* (Hs.PT.56a.25562421.g). "Relative mRNA expression" levels were calculated and normalized to 18s rRNA (Cat#4308329, Applied Biosystems), as described previously (22).

Antibodies and reagents

The following antibodies were used for Western blots: anti-β-actin clone 8H10D10 ab#3700, anti-PARP ab#9542 (Cell Signaling Technology); anti-SULT2B1 ab88085 (Abcam); anti-AR clone 441, anti-β-tubulin T0198, and goat anti-mouse IgG-HRP (Santa-Cruz Biotechnology); anti-human PSA A0562 (Dako); and goat anti-rabbit IgG-HRP (Vector Laboratories). The approximate molecular weight of each protein is indicated with blots in relevant figures.

Caspase-3 activity was measured after 72 hours of siRNA transfection using the Caspase-Glo 3/7 assay (Promega) and analyzed using a Luminoskan Ascent microplate luminometer (Thermo Scientific) according to the manufacturer's instructions. CellTiter 96 AQ$_{ueous}$ One Solution Reagent (Promega) was used for MTS cell proliferation assays, and relative absorbance was quantified by a Multiskan FC plate reader (ThermoScientific). For relevant assays, 20 μmol/L of pan-caspase inhibitor Z-VAD-FMK (Promega) and/or 10 μmol/L of Necrostatin-1 (Sigma) was added to cells at the time of siRNA transfection. Synthetic androgen, R1881 (Sigma), was used at the indicated concentrations.

Production of DU 145-SULT2B1b and LNCaP-SULT2B1b cells

To produce cell lines with tetracycline-inducible expression of SULT2B1b, consecutive lentiviral transductions were performed. DU 145 or LNCaP cells were transduced with lentivirus produced from the Lenti-X Tet-On Advanced Inducible Expression System (Clontech) and then from the pLVX-Tight-Puro lentiviral vector (Clontech) containing human SULT2B1b cDNA. The SULT2B1b cDNA transcript was obtained from Dr. Charles Falany (University of Alabama). Stable selection of both transductions resulted in DU 145-SULT2B1b or LNCaP-SULT2B1b cells. Additional information is available in Supplementary Materials and Methods.

Soft-agar assay

LNCaP cells were transfected with shRNA plasmids in 60-mm dishes for 24 hours and harvested. Soft-agar plates were prepared as described in Supplementary Materials and Methods. Each layer received 1 μg/mL doxycycline for induction of Tet-ON shRNA plasmids. Plates were incubated for 7 days and then cell colonies were counted.

Flow cytometry: cell-cycle analysis and annexin V staining

Treated cells were harvested and then washed twice with PBS, fixed in 75% ethanol, and stored at −20ºC for up to 7 days. To

prepare for flow cytometry, cells were stained with PI (Biolegend #421301) and, in relevant assays, Annexin V (Biolegend #640920) according to the manufacturer's instructions. Staining buffer details are indicated in Supplementary Materials and Methods. Cells were stained at room temperature before analysis on FACS-Canto II (BD Biosciences). Further analysis of DNA content was completed via FlowJo software version 9 (TreeStar Inc.).

Luciferase assays and reporter constructs

AR activity was measured by luciferase assay, as described previously (23). SULT2B1b or AR overexpression was conducted transiently under a CMV promoter. Briefly, LNCaP cells were transfected with shRNA/siRNA, pRL-TK (Promega), and a construct controlled by the AR-responsive portion of the PSA promoter driving firefly luciferase (23). Assays with shRNA KD constructs were transfected simultaneously with luciferase plasmids using Fugene HD (Promega). Assays with siRNA KD were first transfected with luciferase plasmids using Fugene HD for 8 to 16 hours, and then media were changed for consecutive transfection with siRNA as described above. After 24 hours, RNAi transfection, 1 nmol/L R1881 (Sigma) or ethanol control was added to respective wells and incubated for an additional 24 hours. Then, cell lysates were tested for Firefly and *Renilla* luciferase activity using the Dual Luciferase Reporter Assay kit (Promega; ref. 24) and relative luciferase activity (RLU = Firefly/*Renilla*) of the AR is shown as mean ± SEM from at least three independent experiments performed in triplicate.

LXR activity was determined by transfecting LNCaP cells with LXR-RE or Negative Control plasmids (Qiagen CCS-0041L) along with Control or SULT2B1b siRNA. After indicated time of transfection, cells were lysed and analyzed as above. Data were interpreted by determining RLU and then further normalizing samples containing the LXR-RE reporter to samples transfected with the Negative Control reporter.

Statistical analysis

Data were represented as the mean ± standard error of the mean. Statistical analysis was performed using the unpaired two-tailed student's *t* test or two-way ANOVA and analyzed by GraphPad Prism 5 or SAS Enterprise.

Results

CS accumulation correlates with SULT2B1b expression in prostate cancer cells and human tissue specimens

We previously demonstrated that CS accumulation occurs within PIN and prostate cancer tissues compared with normal prostate tissues (10). As an extension of the previous work, a small subset of samples (12 samples ranging from normal to high-grade carcinoma obtained from 8 different patients) was randomly selected from the Indiana University Simon Cancer Center Tissue Bank for comparison of the colocalization of SULT2B1b staining by IHC and areas of CS accumulation by DESI-MS. These studies showed that areas with CS detection were limited to regions of positive SULT2B1b expression (Fig. 1A; Supplementary Fig. S1). To study the role of SULT2B1b *in vitro*, human prostatic epithelial cell lines were analyzed for SULT2B1b expression and activity. Androgen responsive cell lines LNCaP, VCaP, RWPE-1, and the castration nonresponsive line C4-2 generally express higher levels of SULT2B1b than AR− cell lines, such as PC-3 and DU 145 cells

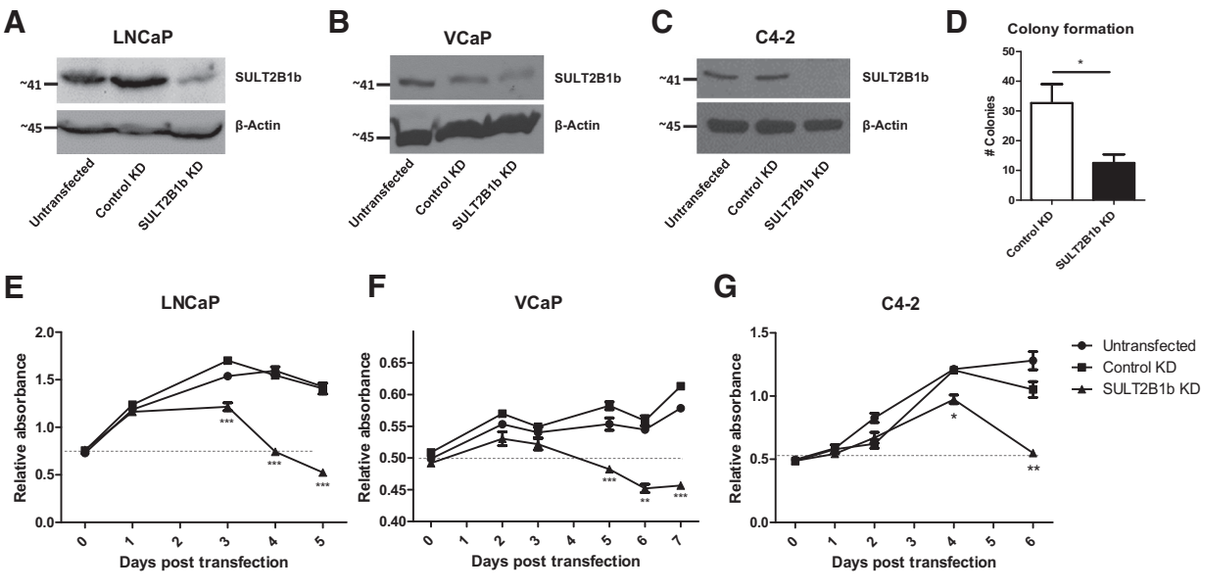

Figure 2.

Targeted KD of SULT2B1b impairs growth/viability of prostate cancer cells. **A–C,** Western blot of SULT2B1b protein expression in LNCaP (**A**), VCaP (**B**), and C4-2 (**C**) cells after a 72-hour incubation with nontargeting (control), or SULT2B1b siRNA. **D,** LNCaP cells were transfected with scrambled control or SULT2B1b shRNA vector. After 24 hours, cells were split into 96-well plates and allowed to grow for 7 days before counting the number of colonies. *, $P < 0.05$. Data represent mean ± SEM of three independent experiments. **E–G,** cell viability/growth curves in LNCaP (**E**), VCaP (**F**), and C4-2 (**G**) cells with treatment of control or SULT2B1b siRNA on day 0. Cell viability was measured via MTS assay at indicated time points. Each point represents the mean ± SEM of 3–4 duplicate wells from each sample, and growth curves are representative of three independent experiments. Statistics were completed at each time point using a Student *t* test with Bonferroni correction. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with control KD cells.

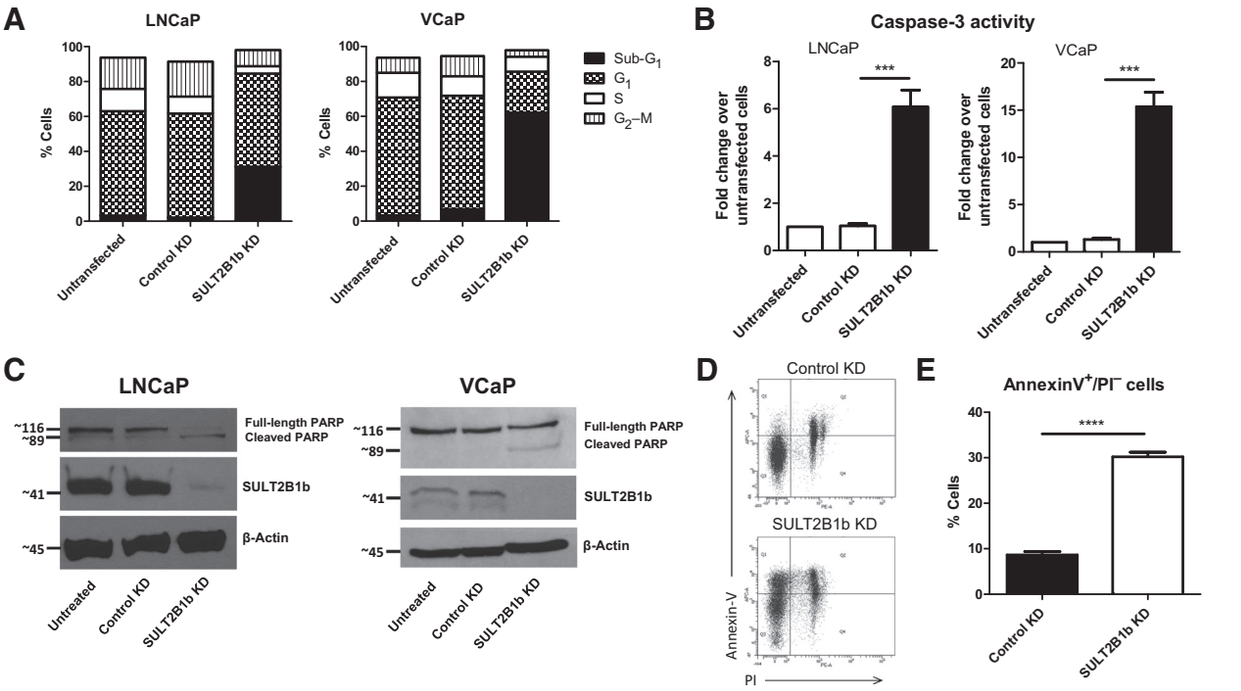

Figure 3.

SULT2B1b KD induces cell death in prostate cancer cells. LNCaP and VCaP cells were transfected with control or SULT2B1b siRNA and harvested after 72 hours. **A,** cell-cycle analysis of LNCaP (left) and VCaP (right) cells. Cell samples were analyzed by flow cytometry after fixing and staining with propidium iodide (PI). Data are representative of three independent experiments. **B,** caspase-3 activity in LNCaP (left) and VCaP (right) cells was determined by luminescence assay after control or SULT2B1b siRNA KD. Results indicate the mean ± SEM of three independent experiments. ***, $P < 0.001$. **C,** Western blots of indicated proteins after control or SULT2B1b siRNA transfection in LNCaP (left) and VCaP (right) cells. **D,** a representative sample of LNCaP cells after incubation with control or SULT2B1b siRNA Annexin V and PI. Cells were harvested and stained before analysis by flow cytometry. **E,** summary of replicate samples shown in **D**. Data represent the percentage of Annexin V^+/PI^- cells out of the total population of events (excluding doublets) and are representative of three independent experiments performed in duplicate. ****, $P < 0.0001$.

(Fig. 1B and C). In general, SULT2B1b expression in these cell lines corresponds to detection of CS, indicating functional activity of SULT2B1b (Fig. 1D). However, it is noteworthy that SULT2B1b can be expressed but not active, as in PC-3 cells (Fig. 1B–D). To demonstrate that CS production was the result of SULT2B1b activity specifically, expression of SULT2B1b was induced in cells that normally express a minimal level of SULT2B1b. A stable, doxycycline-inducible DU 145-SULT2B1b cell line was developed to overexpress SULT2B1b. Induction of SULT2B1b expression by doxycycline resulted in a striking increase in the relative abundance of CS (Fig. 1E and F). Similarly, inducing SULT2B1b overexpression in doxycycline-inducible LNCaP-SULT2B1b cells resulted in an increase in the relative abundance of CS (Supplementary Fig. S2A). Conversely, SULT2B1b KD in LNCaP cells decreased the relative abundance of CS (Supplementary Fig. S2B). While not quantitative, these DESI-MS observations provide strong correlative evidence that SULT2B1b activity is responsible for the accumulated CS in prostate cancer.

Targeted KD of SULT2B1b impairs growth/viability of prostate cancer cells

In order to determine if SULT2B1b affects prostate cancer through production of its product, CS, exogenous CS was added to LNCaP and observed for alterations in phenotype and growth characteristics. Notably, no overt phenotypic changes in growth or cell death were observed (data not shown). Similarly, overexpression of SULT2B1b in LNCaP cells did not enhance growth (data not shown). Thus, RNAi-mediated KD of SULT2B1b was performed to better understand the function of this enzyme in prostate cancer cells. SULT2B1b KD resulted in a decrease in growth/viability of LNCaP, VCaP, C4-2, and RWPE-1 cells as well as decreased soft-agar colony formation in LNCaP cells (Fig. 2; Supplementary Fig. S3). Importantly, a significant decrease in cell growth/viability was observed using multiple SULT2B1b RNAi sequences in LNCaP cells (Fig. 2A, D and E; Supplementary Fig. S4A).

SULT2B1b KD induces cell death in prostate cancer cells

Further investigations showed that SULT2B1b KD increased the percentage of sub-G_1 nuclei by cell-cycle analysis and significantly increased caspase-3 activity and PARP cleavage in both LNCaP and VCaP cells (Fig. 3A–C). Increased sub-G_1 nuclei percentages and caspase-3 activation was also observed in C4-2 cells with SULT2B1b KD (Supplementary Fig. S5). Furthermore, LNCaP cells with SULT2B1b KD analyzed by flow cytometry showed an increased percentage of Annexin V^+/propidium iodide $(PI)^-$ cells compared with control KD cells (Fig. 3D and E).

To determine whether caspase activation was an essential aspect of the induced cell death, abrogation of apoptosis by the addition of a pan-caspase inhibitor, Z-vad-fmk (Z-vad), was tested in SULT2B1b KD LNCaP cells. Results indicated that Z-vad

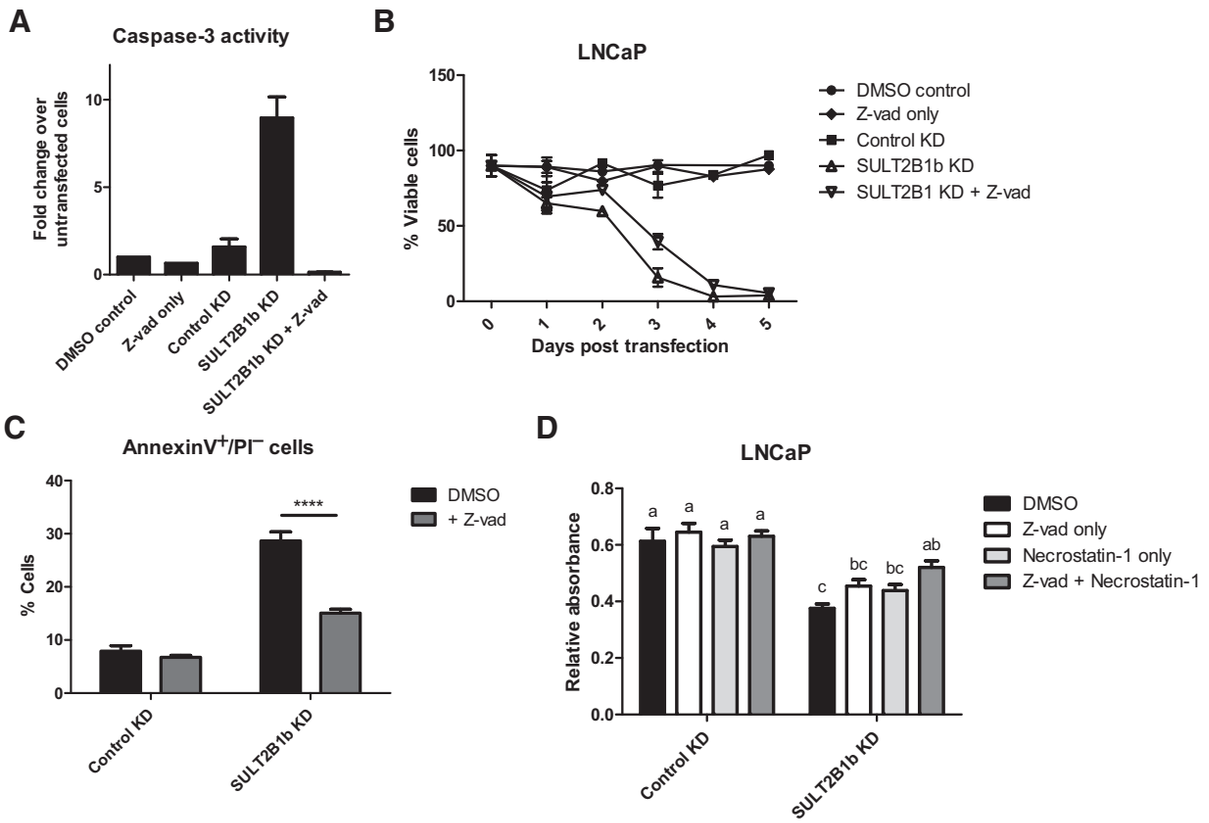

Figure 4.

Cell death resulting from SULT2B1b KD persists with pan-caspase inhibition. **A,** caspase-3 activity in LNCaP cells at 72 hours after transfection. Bars indicate the mean ± SEM of duplicate experiments. **B,** cell viability determined via trypan blue cell counting in LNCaP cells at indicated time points after control or SULT2B1b siRNA KD with and without addition of 20 μmol/L pan-caspase inhibitor, Z-vad. **C,** LNCaP cells were treated with 20 μmol/L Z-vad at the time of control or SULT2B1b siRNA transfection. After 72 hours, cells were harvested, incubated with Annexin V and PI, and analyzed by flow cytometry. Bars represent the mean ± SEM of percentage Annexin V^+/PI^- cells within total events collected (excluding doublets) from three independent experiments. **D,** graph of MTS assay endpoint analysis in LNCaP cells after 96 hours of control or SULT2B1b siRNA KD as well as treatment with 20 μmol/L Z-vad and/or 10 μmol/L Necrostatin-1. Z-vad was added every 24 hours and Necrostatin-1 was added every 48 hours in applicable samples. Bars represent the mean ± SEM of three independent experiments. Statistical analysis was conducted using two-way ANOVA and Tukey posttest. Different letters indicate significant differences among treatments.

successfully blocked caspase-3 activation and abrogated the enhancement of Annexin V^+/PI^- cells after SULT2B1b KD, but LNCaP cell viability and, ultimately, cell death was not altered (Fig. 4A–C). While the complete mechanisms of cell death are not understood, it is possible that after pan-caspase inhibition, SULT2B1b KD cells activated alternative cell death pathways. Additional studies completed in SULT2B1b KD cells with cotreatment of Z-vad and an additional inhibitor of RIP1 kinase, Necrostatin-1, yielded a greater proportion of viable cells compared with Z-vad treatment alone (Fig. 4D). It may be of note that although LNCaP cells with SULT2B1b KD respond to Necrostatin-1 treatment with concurrent pan-caspase inhibition, our studies investigating reactive oxygen species production and Annexin V^-/PI^+ cells did not show that RIP1 kinase-dependent death pathways (i.e., during necroptosis; ref. 25) are directly induced by SULT2B1b KD (data not shown).

SULT2B1b activity modulates AR activity in prostate cancer cells

Prostate cancer cells rely on AR activity for growth and stimulation, and previous studies have demonstrated that cholesterol can be used as a precursor for androgen synthesis (6, 26). Thus, the impact of SULT2B1b modulation on AR expression and activity was evaluated. The data show that SULT2B1b KD by various methods in LNCaP cells decreases AR expression as well as AR activity measured by both prostate specific antigen (PSA) expression and transcription of the AR-response element (AR-RE) within the PSA promoter (Fig. 5; Supplementary Fig. S4B–S4C).

Further investigation showed that the decreased expression of the AR mediated by SULT2B1b KD was not the cause of decreased cell growth, because transient overexpression of the AR in LNCaP cells with SULT2B1b KD showed no increase in cell growth over LNCaP cells with SULT2B1b KD alone (Fig. 6A–C). Endpoint analysis of a growth assay in LNCaP cells shows that replenishing the culture medium with synthetic androgen, R1881, partially, yet significantly, rescued the reduced cell growth in SULT2B1b KD LNCaP cells back to control KD levels (Fig. 6D).

SULT2B1b regulates the AR independently of LXR

Transient overexpression of the human SULT2B1b cDNA was performed to address whether or not SULT2B1b activity regulates

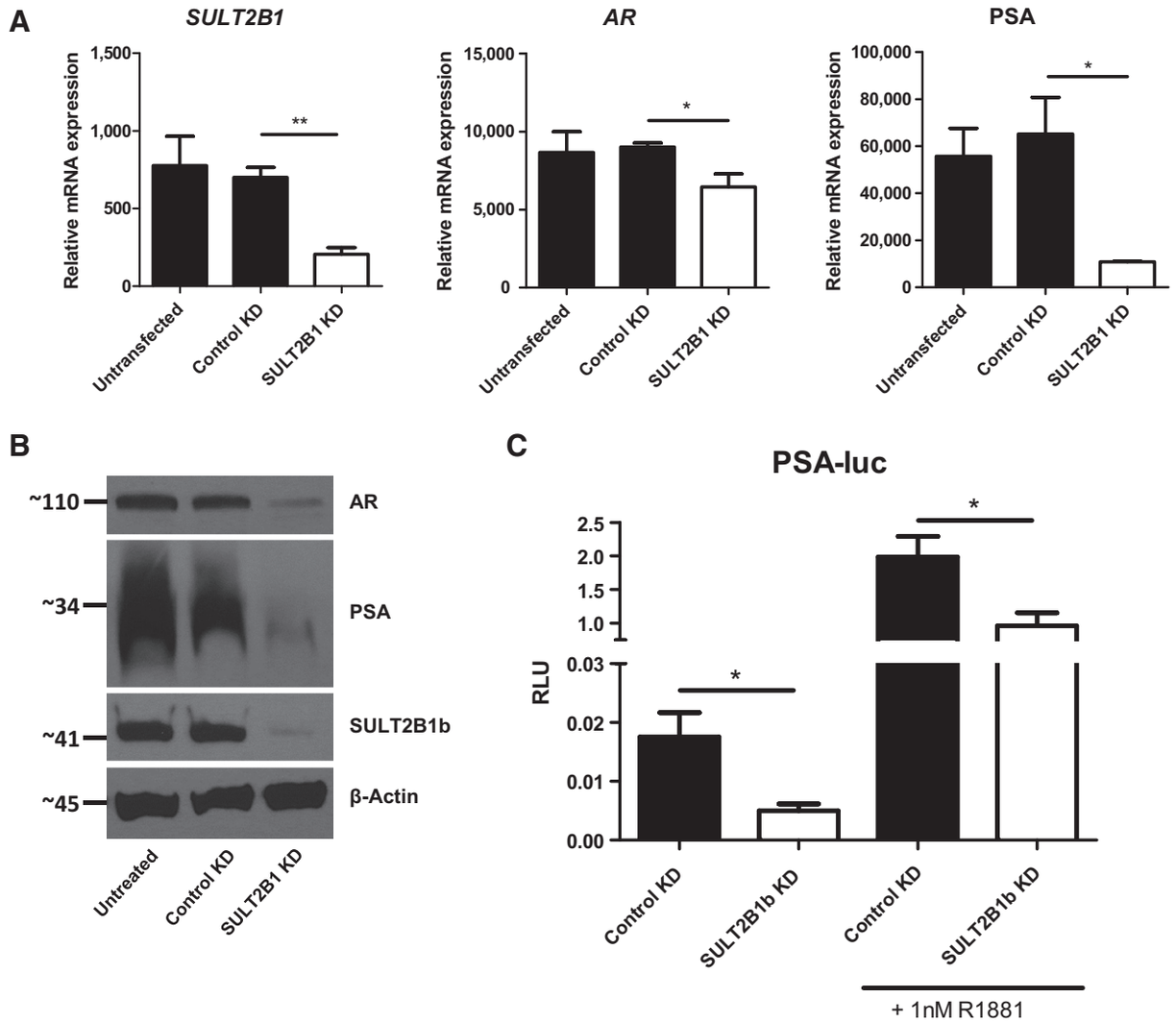

Figure 5.

SULT2B1b KD decreases AR expression and activity. **A** and **B,** LNCaP cells treated with control or SULT2B1b siRNA were harvested at 72 hours for mRNA expression by qRT-PCR (**A**) and protein by Western blot (**B**) of indicated genes and proteins, respectively. **C,** a luciferase reporter construct controlled by the AR-responsive portion of the PSA promoter was transfected into LNCaP cells followed by transfection with control or SULT2B1b siRNA. Cells were harvested and lysed 48 hours after siRNA transfection. Firefly luciferase activity was normalized to *Renilla*. Relative luciferase units (RLU) are shown for cell conditions with or without addition of 1 nmol/L R1881 added 24 hours before harvest. Bars represent the mean ± SEM of three independent experiments. *, $P < 0.05$ determined by the *t* test.

LXR activity in prostate cancer cells (27). LNCaP cells with SULT2B1b overexpression (hSULT2B1b vector) showed a significant decrease in LXR activity and a trend of decreased transcription of downstream target gene, ATP-binding cassette (ABC)-G1 (Fig. 7A–C).

Additionally, because LXR activation has been shown to decrease AR activity (28), the question of whether activation of LXR in SULT2B1b KD cells causes decreased AR transcription was addressed. Double siRNA KD of SULT2B1b and LXRβ was performed followed by the assessment of AR activity through PSA expression (Fig. 7D–F). LXRα was excluded because LXR activity in LNCaP cells was demonstrated to be due to transcriptional activation of LXRβ (Fig. 7D; Supplementary Fig. S6). Double KD of SULT2B1b/LXRβ did not affect the siRNA KD efficiency compared with SULT2B1b or LXRβ KD alone (Fig. 7E). Regardless of whether or not LXR was present/active, SULT2B1b KD was still able to inhibit AR activity, as shown by a significant reduction in PSA expression (Fig. 7F).

Discussion

We previously reported that CS accumulates in precancerous and cancerous human prostate specimens (10). To date, the role of CS in the prostate or the impact of accumulated CS within prostatic cells is not known. Data in this study utilized DESI-MS technology to show that CS accumulation is the result of increased

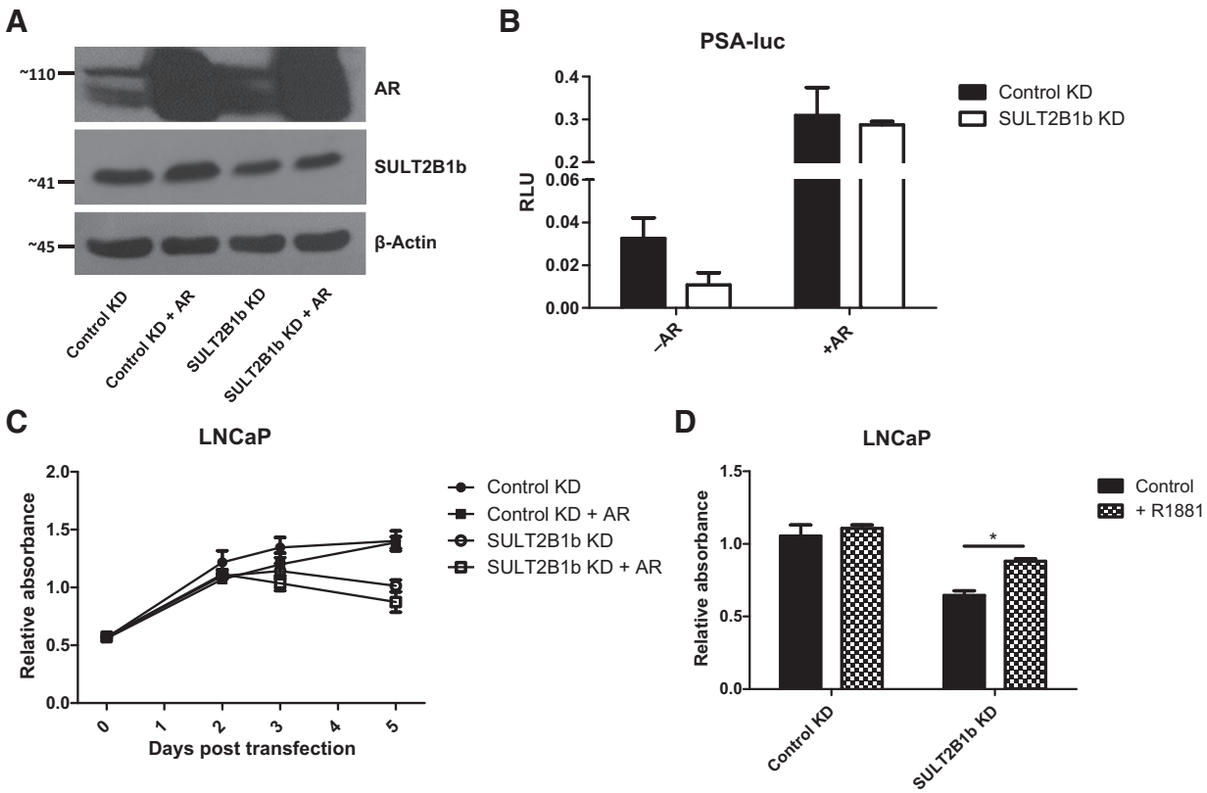

Figure 6.

AR ligand addition, but not AR overexpression, partially rescues cell growth in LNCaP with SULT2B1b KD. **A,** Western blot showing expression levels of indicated proteins with control or SULT2B1b KD ± AR overexpression. Cells were harvested 60 hours after transfection with control or SULT2B1b siRNA. **B,** luciferase assay showing AR activity after 72 hours of siRNA transfection. **C,** MTS assay of LNCaP cells with or without AR overexpression followed by control or SULT2B1b siRNA transfection. The time of siRNA transfection was considered Day 0 and relative absorbance was identified at the indicated time points. Each point represents the mean ± SEM of 3 replicate wells. **D,** 72-hour endpoint analysis from an MTS assay of LNCaP cells that were transfected with control or SULT2B1b siRNA with or without addition of 10 nmol/L R1881 daily. Each bar represents the mean ± SEM of 3 replicate wells, and similar results were found in duplicate experiments. *, $P < 0.05$, determined by two-way ANOVA.

SULT2B1b activity, rather than simply expression, within prostate cells (Fig. 1; Supplementary Fig. S1). Detection of accumulated CS at the macroscopic level reveals either enhanced activity of SULT2B1b or an inability of the tissue to secrete or utilize this product. Nonetheless, the use of DESI-MS in these studies allowed the validation that manipulation of SULT2B1b in cell lines led to functional alterations (Fig. 1E and F; Supplementary Fig. S2).

Mechanisms of SULT2B1b function other than sulfonation (15, 16) have not been well described. In contrast to our findings, two groups independently reported that SULT2B1b KD promotes LNCaP cell growth while maintained in androgen-depleted conditions with DHEA supplementation (19, 21). Although we do not fully understand the reasons for this discrepancy, culture medium conditions in growth assays may play a role. While their data support the logical hypothesis that SULT2B1b-mediated sulfonation causes "inactivation" of steroid precursors (i.e., DHEA) leading to decreased AR-dependent proliferative signaling, data herein directly show that SULT2B1b activity is positively correlated to AR activity, which implies that an alternative mechanism of AR regulation is occurring. While our studies in cell lines support our previous findings that CS accumulates in precancerous and cancerous prostate tissues compared with normal counterparts, we also found that a single benign cell line, RWPE-1, has robust SULT2B1b expression and activity, which likely reflects the heterogeneity of SULT2B1b expression/activity within prostate tissue, as has been reported by others (19). In contrast to the findings of Seo and colleagues (19), recent evidence in cancer cells suggests SULT2B1b plays a role in cancer growth/aggressiveness. It has been demonstrated that SULT2B1b activity increases gastric cancer angiogenesis and tumor volume, SULT2B1b activity promotes hepatocellular carcinoma cell growth *in vitro* and *in vivo*, and SULT2B1b expression correlates with poor prognosis and promotes tumor cell growth in colorectal cancer patients (17, 18, 20). In fact, the Human Protein Atlas database (http://www.proteinatlas.org/ENSG00000088002-SULT2B1/cancer) of tissue samples supports elevated expression levels of SULT2B1b in prostate cancer compared with normal tissue. As a result of the controversial role of SULT2B1b activity, additional studies in prostate cancer are required to fully understand its function.

We hypothesize that SULT2B1b continues to be essential for prostate cancer cell growth throughout progression of the disease, as our data demonstrate that SULT2B1b KD induces cell death in both androgen-dependent and CRPC cell lines (Figs. 2–4; Supplementary Fig. S5). However, it remains to be determined

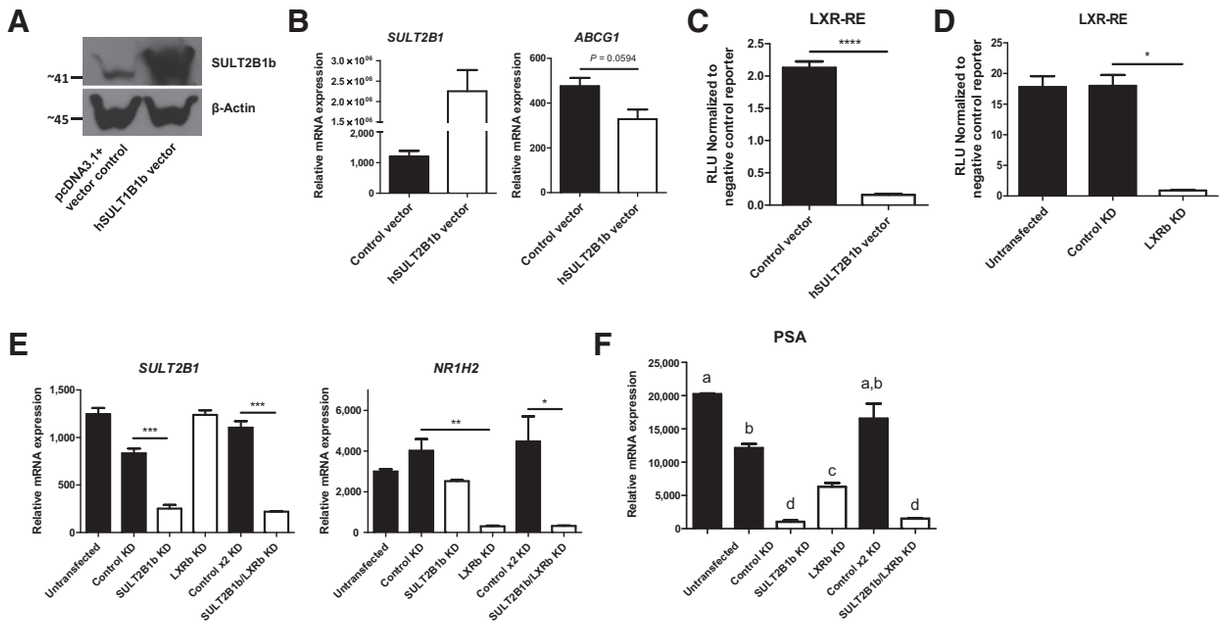

Figure 7.

SULT2B1b regulates the AR independently of LXR. **A–C,** LNCaP cells were transiently transfected with a control vector (pcDNA3.1) or a construct containing the human SULT2B1b cDNA under the CMV promoter (hSULT2B1b vector), resulting in SULT2B1b overexpression. Western blot (**A**) or qRT-PCR (**B**) was performed 72 hours after transfection. Gene expression was normalized to 18S rRNA. Bars represent the mean ± SEM of triplicate samples. **C,** an LXR-responsive luciferase reporter construct under the minimum SV40 promoter was transfected into LNCaP cells with basal expression (control vector) or overexpression (hSULT2B1b vector) of SULT2B1b. Firefly luciferase activity was normalized to *Renilla*. LXR activity was measured and further normalized to negative control luciferase reporter activity for each sample. **D,** LNCaP cells were treated with control or LXRβ siRNA and analyzed for LXR activity via luciferase assay after 72 hours. RLU was quantified and normalized as described in **C**. *T* tests were completed in **B–D** to determine significant differences. *, $P < 0.05$; ****, $P < 0.0001$, compared with control cells. **E** and **F,** double siRNA KD of LXRβ and SULT2B1b was conducted. Bars represent the mean ± SEM of triplicate samples. **E,** SULT2B1b and LXRβ expression determined via qRT-PCR. Gene expression was normalized to 18S rRNA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with control/controlx2 KD cells by one-way ANOVA. **F,** AR activity was evaluated through PSA expression by qRT-PCR. Gene expression was normalized to 18S rRNA. Statistical analysis was conducted using a one-way ANOVA and Tukey posttest. Different letters indicate significant differences among treatments.

whether SULT2B1b activity influences the progression of androgen-dependent prostate cancer cells toward a castration nonresponsive state. Notably, our past investigation of human clinical prostate cancer specimens did not find any correlation between CS accumulation and prostate cancer stage/grade (10).

Cell death induced by SULT2B1b KD includes caspase-3 activation, but pan-caspase inhibition does not reduce cell death overall as it appears that other death mechanisms, such as RIP1 kinase-related pathways, can become activated (Fig. 4B and D). All of these data suggest that KD of SULT2B1b in prostate cancer cells alters cellular metabolism that results in cell death, perhaps by a variety of pathways. Although an exact mechanism of how this cholesterol sulfotransferase could be linked to cell viability was not defined here, the data supporting that SULT2B1b-mediated modulation of AR activity likely plays a role.

SULT2B1b activity has been shown to decrease activity of the LXR through inactivation of endogenous oxysterol ligands and we showed similar results in prostate cancer cells (Fig. 7A–C; ref. 16). Additionally, chemical activation of the LXR with agonist TO901317 has been shown to induce cell death in LNCaP cells grown in serum-free medium (21). We observed that SULT2B1b KD-induced activation of the LXR was minimal compared with direct activation by TO901317 (data not shown), suggesting that SULT2B1b KD in complete medium conditions may endogenously regulate LXR in addition to alternative pathways that culminate in a cell death phenotype.

A possible alternative would be that SULT2B1b KD induces cell death through its impact on AR expression and activity (Fig. 5; Supplementary Fig. S4B–C; ref. 29). However, this may not be due to reduced AR expression levels because overexpression of the AR with SULT2B1b KD does not rescue the cell death phenotype (Fig. 6A–C). Additionally, even though some of the included cell lines do express AR variants, we do not suspect that SULT2B1b function relies completely on variant forms because SULT2B1b KD decreases viability of a range of cell lines, including benign RWPE-1 cells (Supplementary Fig. S3; refs. 30, 31). However, it is not known whether SULT2B1b modulation is capable of affecting activity of AR variants. Perhaps SULT2B1b KD influences transcriptional coregulators of the AR, causing this decrease in activity. LXR activation has also been shown to decrease AR activity in LNCaP cells as well as inhibit androgen-dependent proliferation in a SULT2A1-dependent manner (28). Our data suggest that the impact of SULT2B1b on AR activity is independent of LXR activity, because AR activity decreases regardless of the presence of LXR (Fig. 7D–F). Due to these findings, it is likely that other novel mechanisms of SULT2B1b influence on AR activity are at play. Given that *de novo* androgen synthesis occurs within prostate cancer cells (32), it is also possible that SULT2B1b activity is linked to synthesis of DHT within these cells because the

addition of the AR ligand R1881 abrogates the growth-inhibitory effect of SULT2B1b KD (Fig. 6D).

This is the first study to demonstrate that SULT2B1b modulation alters AR activity. Interestingly, in preliminary RNA-sequencing studies, genes modulated by SULT2B1b KD significantly altered 4 out of 5 overrepresented CRPC-related pathways identified by Robinson and colleagues (33). Regulation of c-myc, cross-talk with the PI3K/Akt pathway, and impact on a number of other proliferation pathways can lead to the formation of CRPC in an androgen-deprived environment (34–36). It may be important to determine the impact of SULT2B1b modulation on these pathways to better understand whether SULT2B1b aids in progression of prostate cancer toward a castration nonresponsive state. Because CRPC retains AR activity in the context of human prostate cancer (26), it is tempting to speculate that SULT2B1b modulation could affect AR activity in this disease stage. Based on our data, it is clear that SULT2B1b is a critical regulator of prostate cancer growth and that SULT2B1b could be the target of a promising anticancer therapeutic that would simultaneously activate LXR and inhibit AR activity. These data and further studies may indicate that SULT2B1b is a significant and novel metabolic target for treatment of human prostate cancer at multiple stages of the disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Cancer Facts and Figures 2016. Available from: http://www.cancer.org/acs/groups/content/@research/documents/document/acspc-047079.pdf.
2. James ND, Spears MR, Clarke NW, Dearnaley DP, De Bono JS, Gale J, et al. Survival with newly diagnosed metastatic prostate cancer in the docetaxel era: Data from 917 patients in the control arm of the STAMPEDE Trial (MRC PR08, CRUK/06/019). Eur Urol 2015;67:1028–38.
3. Ryan CJ, Smith MR, Fizazi K, Saad F, Mulders PFA, Sternberg CN, et al. Abiraterone acetate plus prednisone versus placebo plus prednisone in chemotherapy-naive men with metastatic castration-resistant prostate cancer (COU-AA-302): final overall survival analysis of a randomised, double-blind, placebo-controlled phase 3 study. Lancet Oncol 2015;16:152–60.
4. Scher HI, Fizazi K, Saad F, Taplin M-E, Sternberg CN, Miller K, et al. Increased survival with enzalutamide in prostate cancer after chemotherapy. N Engl J Med 2012;367:1187–97.
5. Locke JA, Nelson CC, Adomat HH, Hendy SC, Gleave ME, Guns EST. Steroidogenesis inhibitors alter but do not eliminate androgen synthesis mechanisms during progression to castration-resistance in LNCaP prostate xenografts. J Steroid Biochem Mol Biol 2009;115:126–36.
6. Mostaghel Ea, Solomon KR, Pelton K, Freeman MR, Montgomery RB. Impact of circulating cholesterol levels on growth and intratumoral androgen concentration of prostate tumors. PLoS One 2012;7:e30062.
7. Freeman MR, Solomon KR. Cholesterol and prostate cancer. J Cell Biochem 2004;91:54–69.
8. Freeman MR, Cinar B, Lu ML. Membrane rafts as potential sites of nongenomic hormonal signaling in prostate cancer. Trends Endocrinol Metab 2005;16:273–9.
9. Zhuang L, Kim J, Adam RM, Solomon KR, Freeman MR. Cholesterol targeting alters lipid raft composition and cell survival in prostate cancer cells and xenografts2005;115.
10. Eberlin LS, Dill AL, Costa AB, Ifa DR, Cheng L, Masterson T, et al. Cholesterol sulfate imaging in human prostate cancer tissue by desorption electrospray ionization mass spectrometry. Anal Chem 2010;82:3430–4.
11. Falany CN, Comer KA, Dooley TP, Glatt H. Human dehydroepiandrosterone sulfotransferase. Ann N Y Acad Sci 1995;774:59–72.
12. Falany CN, He D, Dumas N, Frost AR, Falany JL. Human cytosolic sulfotransferase 2B1: isoform expression, tissue specificity and subcellular localization. J Steroid Biochem Mol Biol 2006;102:214–21.
13. Higashi Y, Fuda H, Yanai H, Lee Y, Fukushige T, Kanzaki T, et al. Expression of cholesterol sulfotransferase (SULT2B1b) in human skin and primary cultures of human epidermal keratinocytes. J Invest Dermatol 2004;122:1207–13.
14. He D, Meloche CA, Dumas NA, Frost AR, Falany CN. Different subcellular localization of sulphotransferase 2B1b in human placenta and prostate. Biochem J 2004;379:533–40.
15. Strott CA. Sulfonation and molecular action. Endocr Rev 2002;23:703–32.
16. Chen W, Chen G, Head DL, Mangelsdorf DJ, Russell DW. Enzymatic Reduction of Oxysterols Impairs LXR Signaling in Cultured Cells and the Livers of Mice. Cell Metab 2007;5:73–9.
17. Yang X, Xu Y, Guo F, Ning Y, Zhi X, Yin L, et al. Hydroxysteroid sulfotransferase SULT2B1b promotes hepatocellular carcinoma cells proliferation in vitro and in vivo. PLoS One 2013;8:e60853.
18. Hu L, Yang G-Z, Zhang Y, Feng D, Zhai Y-X, Gong H, et al. Overexpression of SULT2B1b is an independent prognostic indicator and promotes cell

growth and invasion in colorectal carcinoma. Lab Investig 2015;95: 1005–18.
19. Seo Y-K, Mirkheshti N, Song CS, Kim S, Dodds S, Ahn SC, et al. SULT2B1b sulfotransferase: induction by vitamin D receptor and reduced expression in prostate cancer. Mol Endocrinol 2013;27:925–39.
20. Chen W, Zhou H, Ye L, Zhan B. Overexpression of SULT2B1b promotes angiogenesis in human gastric cancer. Cell Physiol Biochem 2016;38: 1040–54.
21. He D, Falany CN. Inhibition of SULT2B1b expression alters effects of 3 b -hydroxysteroids on cell proliferation and steroid hormone receptor expression in human LNCaP prostate cancer cells. Prostate 2007;67: 1318–29.
22. Burcham GN, Cresswell GM, Snyder PW, Chen L, Liu X, Crist SA, et al. Impact of prostate inflammation on lesion development in the POET3(+) Pten(+/-) mouse model of prostate carcinogenesis. Am J Pathol 2014; 184:3176–91.
23. Hsu C-C, Hu C-D. Transcriptional activity of c-Jun is critical for the suppression of AR function. Mol Cell Endocrinol 2013;372:12–22.
24. Hsu C-C, Hu C-D. Critical role of N-terminal end-localized nuclear export signal in regulation of activating transcription factor 2 (ATF2) subcellular localization and transcriptional activity. J Biol Chem 2012;287:8621–32.
25. Degterev A, Zhou W, Maki JL, Yuan J. Assays for Necroptosis and activity of RIP kinases. Methods Enzymol 2014;545:1–33.
26. Heinlein CA, Chang C. Androgen receptor in prostate cancer. Endocr Rev 2004;25:276–308.
27. Bai Q, Xu L, Kakiyama G, Runge-Morris MA, Hylemon PB, Yin L, et al. Sulfation of 25-hydroxycholesterol by SULT2B1b decreases cellular lipids via the LXR/SREBP-1c signaling pathway in human aortic endothelial cells. Atherosclerosis 2011;214:350–6.
28. Lee JH, Gong H, Khadem S, Lu Y, Gao X, Li S, et al. Androgen deprivation by activating the liver X receptor. Endocrinology 2008;149:3778–88.
29. Eder IE, Culig Z, Ramoner R, Thurnher M, Putz T, Nessler-Menardi C, et al. Inhibition of LncaP prostate cancer cells by means of androgen receptor antisense oligonucleotides. Cancer Gene Ther 2000;7:997–1007.
30. Hu R, Dunn TA, Wei S, Isharwal S, Veltri RW, Humphreys E, et al. Ligand-independent androgen receptor variants derived from splicing of cryptic exons signify hormone-refractory prostate cancer. Cancer Res 2009;69: 16–22.
31. Guo Z, Yang X, Sun F, Jiang R, Linn DE, Chen H, et al. A novel androgen receptor splice variant is up-regulated during prostate cancer progression and promotes androgen depletion-resistant growth. Cancer Res 2009;69: 2305–13.
32. Locke J a, Guns ES, Lubik A a, Adomat HH, Hendy SC, Wood C a, et al. Androgen levels increase by intratumoral de novo steroidogenesis during progression of castration-resistant prostate cancer. Cancer Res 2008;68: 6407–15.
33. Robinson D, Van Allen EM, Wu Y-M, Schultz N, Lonigro RJ, Mosquera J-M, et al. Integrative clinical genomics of advanced prostate cancer. Cell 2015;161:1215–28.
34. Bernard D, Pourtier-Manzanedo A, Gil J, Beach DH. Myc confers androgen-independent prostate cancer cell growth. J Clin Invest 2003;112: 1724–31.
35. Karantanos T, Corn PG, Thompson TC. Prostate cancer progression after androgen deprivation therapy: mechanisms of castrate resistance and novel therapeutic approaches. Oncogene 2013;32:5501–11.
36. Carver BS, Chapinski C, Wongvipat J, Hieronymus H, Chen Y, Chandarlapaty S, et al. Reciprocal feedback regulation of PI3K and androgen receptor signaling in PTEN-deficient prostate cancer. Cancer Cell 2011;19:575–86.